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Screening and Identification of Heavy Metal Resistant Endophytic Fungi Isolated from *Solanum nigrum* and Heavy Metal Impact on Survivability of Earthworm

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Heavy metals are hazardous for both human health and the environment. In order to create and maintain contamination free soil, many efforts are made to develop economically feasible technologies. This research article emphasizes on phytoremediation by endophytic fungi (*Alternaria alternata*) isolated from leaves of Solanum nigrum.

Materials and Methods: The endophytic fungus was confirmed by 18S rRNA sequencing. Isolated endophytic fungi was screened individually for heavy metal tolerance at various concentrations to Lead acetate (Pb), Magnesium chloride (Mg), Cadmium sulphate (Cd) and Potassium dichromate (Cr).

Results: The endophytic fungi Alternaria alternate showed greater tolerance against the heavy metal cadmium. The minimum inhibitory activity of the fungi against the heavy metals showed maximum inhibition towards cadmium up to 6000ppm. The metal tolerance index showed maximum growth of Alternaria alternate towards cadmium, followed by other heavy metals Bioaccumulation of heavy metals by the endophytic fungi also demonstrated high tolerance against cadmium (89%). To determine the survivability of earthworm, toxigenicity test was done, the cadmium treated Earthworm was motionless within few minutes after exposure to cadmium when compared with control.

Conclusion: Thus, the study clearly indicates that the endophytic fungi is a boon to control environmental pollution and to retain soil fertility.

Keywords: Alternaria alternate; endophytic fungi; heavy metals; toxigenicity.

ABBREVIATIONS

PCR : Polymerase Chain Reaction
PDB : Potato Dextrose Broth
MIC : Minimum Inhibitory Concentration
MTI : Metal Tolerance Index

1. INTRODUCTION

Quite a few heavy metals have high atomic weights and densities. Most of them are released into the environment and can be found in the biosphere, which includes rocks, soils, and water.[1] By combining with various environmental components including water, soil, and air, heavy metals can become very hazardous, and humans and other living things may be exposed to them through the food chain.[2] Environmental contaminants known for their toxicity, long atmospheric half-lives, and ability to bioaccumulate in human tissue are heavy metals. Vital heavy metals that are naturally occurring enter the body system through food, water and air, where they govern a variety of biological processes.[3]

Two sources through which the heavy metals enter the environment are natural and anthropogenic sources. The Earth's crust contains metals by nature. The natural source of heavy metals is rocks. They can be found in the rocks as minerals such as lead. Agriculture and industry are the primary human-caused sources of heavy metal contamination in the environment. Heavy metals are released into the environment through discharges of wastewater and sewage. Burning fossil fuels and using chemical fertilizers are two more anthropogenic sources of heavy metal pollution in the environment. [4]

Minimum levels of heavy metals are required for health maintenance, but larger concentrations can be toxic or dangerous. Bio magnifications could result from these hazardous heavy metals contaminating the ecosystem.[5] Several heavy metals affect biological processes and development, while others build up in one or more organs and cause a number of serious illnesses like cancer, diseases of the skin, abnormalities in nervous system, etc. Free radicals are produced as a result of metal poisoning, which harms DNA.[6]

Fungi may detoxify and tolerate metals by a variety of methods, like extracellular precipitation, valence transformation, and active absorption. Through chemical alteration or by impacting chemical bioavailability, fungi possess the ecological and biochemical ability to lessen the risk posed by metals, radionuclides and metalloids. Fungi are ideal for bioremediation procedures because they may form long mycelial networks. The use of filamentous fungi can be a promising strategy when bacteria are unable to produce the mycelia network required to deal with pollutants. [7]

Endophytes are referred to as microbes that invade a plant's internal tissues without having an adverse impact. Endophytes have been thoroughly investigated in a wide range of geographic and climatic zones, and it has been discovered that they are present in all investigated plants till date. There are many different orders, families, genera, and species present in the host [8]. Through the root zone, flower, leaf, and cotyledon, endophytes frequently enter plant tissue where they can either stay localized or spread throughout the entire plant. Depending on their mode of survival, endophytes can be classified as "obligate" or "facultative." Obligate endophytes can only develop and survive on the host plant, and they can spread to other plants either vertically or by vectors. Facultative endophytes go through a phase in which they don't live inside host plants. Complex chemicals can be easily broken down by endophytes [9].

Plants typically exhibit toxicity from heavy metals, although many also exhibit metal tolerance and hyperaccumulation [10,11] Phytoremediation, by endophytic fungi from plants has been put forth as an environmental friendly, and very efficient in eliminating heavy metal toxicants from the polluted soil. Endophytic fungi have the capability to accumulate heavy metals in higher amounts without enhancing phytotoxicity.

Plants have the ability to efficiently hyperaccumulate heavy metals. Many heavy metal resistant endophytic fungi were obtained from plants such as *Solanum nigrum, Brassica napus,Alnus firma,* and *Thlpai caerulescens* [12].

Endophytic fungi play a very crucial role in heavy metal bio absorption from contaminated soil, among them *Aspergillus niger* and *Pencillium spp* exhibit very potential bioabsorption capacities [13].

The primary goal of the research is to isolate a potent endophytic fungus from the plants, examine the fungus's ability to tolerate heavy metals against different metals, and determine toxigenicity of the heavy metal against earthworms.

2. MATERIALS AND METHODS

2.1 Endophytic Fungi Isolation and Identification

Endophytic fungi were isolated from the leaves of *Solanum nigrum* collected from SRM campus, Chennai. The leaves were surface sterilized and air dried in sterile condition. The leaves of *Solanum nigrum* were placed on the sterile PDA plates and incubated at 25° C for 4 to 6 days. The different mycelial growths were transferred to fresh PDA medium to obtain pure culture. The isolated endophytic fungi were characterized by macroscopic and microscopic methods.[14]

2.2 Selection of Heavy Metal Resistant Endophytic Fungi

Heavy metals chosen for the research (Lead, Cadmium, Mercury and Chromium) were prepared in various concentrations, such as 100,200,400, and 600ppm. Isolated endophytic fungi were screened individually with various concentrations of heavy metals and the fungal growth was observed in comparison to the control. The fungal isolates showing higher resistance against various heavy metals were further tested for tolerance by increasing the concentration from 1000ppm, 3000ppm, and 6000ppm. The fungal mycelia was plated in PDA and incubated at 25° C for 4 to 6 days [15,16].

2.3 Molecular Identification of *Alternaria alternata*

2.3.1 DNA isolation

DNA was extracted from the samples using a modified Cetyltrimethylammonium bromide (CTAB) technique.

In a sample tube, 400µL of CTAB solution and a loop full of fungal mate was taken. Using a sterile homogenizer, homogenise the fungus sample. And ProteinaseK 5µL (20 mg/mL) was incorporated. For simple digestion, the mixture was maintained at 55°C in a water bath for 2 hours (with intermittent mixing/quick vortex). The mixture was spun up in a Centrifuge at 12,000 rpm for 10 minutes. After transferring the supernatant to a new tube, an equal volume of chloroform:isoamylalcohol was added. The aqueous layer was transferred to a fresh tube and 0.7 litre of IPA (isopropanol) was added after the tube had been vigorously mixed for 10 minutes at 12,000 rpm. After gently mixing the tube for 15 seconds, it was centrifuged at 12,000 rpm for 10 minutes in order to extract the supernatant, and 500 µL of ethanol 70% was added for washing. For 5 minutes, the sample was spun at 11000 rpm. Remove the supernatant with care and allow to air dry at room temperature. Partially dried DNA was

reconstituted in 50 µL of elution and stored at -20°C.[17]

2.4 DNA Quality Determination

2.4.1 Quantification of DNA

The amount of isolated DNA was measured using the Nano drop 2000 (Thermo, USA). The absorbance ratio at 260 and 280 nm (260/280) was used to verify the quality. A score between 1.7 and 1.8 indicates high-quality DNA free of protein/RNA contamination.

2.4.2 DNA quality determination

DNA quality was determined using 0.8% agarose gels.

2.4.3 Molecular region

The standard ITS region was chosen for molecular verification of the fungal species in this study. Using universal primers, PCR was used to extend the ITS region.

2.4.4 Primers

For full amplification, universal Fungal ITS primers were chosen from earlier investigations [18] (they encompass all variable areas).

2.4.5 Master mix components

- Distilled water - 18 µL
- Taq DNA polymerase (3U/ µL) - 0.33 µL
- dNTP (each 2.5mM) - 2 µL
- Primer mix - 1 µL
- Template DNA - 1 µL
- 10x Mgcl2 Buffer - 2.67 µL

2.4.6 PCR (Polymerase Chain Reaction)

Polymerase chain reaction was carried out at the temperatures and times specified in the Applied Biosystems thermal cycler.

- Initial denaturation 94 ° C for 5 min
- Denaturation 94 ° C for 30 sec
- Annealing 56 ° C for 30 sec 35 Cycles
- Extension 72 ° C for 45 sec
- Final extension 72 ° C for 7 min

On 1.5% Agarose gel electrophoresis, the amplified products were examined, and the molecular weight was determined using a molecular weight marker (100bp ladder).

2.5 Sequencing the Amplified Product

2.5.1 Sequencing using big dye terminator v3.1

The sequencing process was carried out in a PCR thermal cycler (Applied Biosystems' GeneAmp PCR System 9700) with the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA).

2.6 Sequence Analysis

The sequencing process was carried out in a PCR thermal cycler (Applied Biosystems' GeneAmp PCR System 9700) with the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA).

2.7 Blast Analysis

The ITS-related amplified sequences were validated using a similarity index generated by the NCBI's BLAST Programme. The species would be determined based on the highest percentage of resemblance to the reference species.

2.8 Metal Tolerance Index

Endophytic fungi (*Alternaria alternata*) was checked for metal tolerance index. PDA plates supplemented with 50 ppm to 6000ppm of heavy metals, the fungal isolates were inoculated and incubated at 27° C for 4 to 6 days. The fungal growth effect on each metal was estimated by measuring the radius of fungal growth beside the control plate (without heavy metal).Tolerance index was calculated using the formula [19].

> Metal Tolerance index (Ti) = Ratio of the colony treated by fungus to its increased radius Ratio of untreated colony

Percentage inhibition of fungal mycelial growth by heavy metal concentration was calculated using the formula

> Ti= Dt/Du

Dt- radial extension (cm) of treated fungal colony.

Du - radial extension (cm) of untreated colony.

2.9 Determination of MIC (Minimum Inhibitory Concentration)

The heavy metals such as Cadmium (cadmium sulphate), Lead (Lead acetate), Mercury (Mercury Chloride) and Chromium (Potassium di chromate) were supplemented with various concentrations from 50ppm to 6000ppm in potato dextrose agar and fungus agar plug(3mm) was inoculated and incubated at 27° C for 10 days. The MIC of the isolate was determined as the lower concentration of heavy metal that inhibits visible growth of the isolates. Fungal plug inoculated on PDA without metal serves as control [20].

2.9.1 Bioaccumulation of heavy metals

Fungal agar plug (8 days old) was inoculated in 100 ml of PDB(potato dextrose broth) impregnated with 6000 ppm of chromium, further the inoculated broth was incubated at 27° C for 7 days. As a control, heavy metal without a fungal plug was used. After seven days, the fungus growth was obtained using Whatman's filter paper. The filtrate is centrifuged at 12000rpm for 20mins. The heavy metal concentration before and after development of organism was observed by ultra violet spectrometry. Bio accumulation of heavy metal was estimated by the following equation [21].

Metal Removal (%) = (Co-Ct)/Cox100

Co- Initial concentration of heavy metal

Ct- Concentration of metal after incubation

2.9.2 Toxigenicity testing

Toxigenicity test was done as mentioned earlier by Parihar, Kapil et al. [22] with slight variation. The heavy metal Cadmium was used to check the survivability of Earth worm. Earth worms were obtained from fertile land inside SRM campus, Chennai. Aqueous form of heavy metal (600ppm) was prepared in petri dish. Active earthworm was exposed to the heavy metal, where control earth worm was maintained in heavy metal free double distilled water. Overall activity and its response were monitored continuously and compared with control and the observation was recorded.

3. RESULTS AND DISCUSSION

3.1 Endophytic Fungi Isolation and Identification

Most important threat to modern agricultural practice is heavy metal contamination. To reinstate the contaminated site various reclamation techniques were practised, however, they are costly or ineffective to remove contaminants. Under such conditions an innovative microbe based technology called bioremediation is applied to reclaim the soil. The current study mainly focused on isolating and characterising heavy metal resistant endophytic fungi obtained from the leaves of *Solanum nigrum* obtained from SRM campus, Kattankulathur, Chennai. In our current research, we isolated and identified four fungal strains, *Alternaria sp., Aspergillus flavus, Aspergillus niger and Cladosporium* (Fig 1) based on macroscopic and microscopic features.

3.2 Selection of Heavy Metal Resistant Endophytic Fungi

Primary screening of four fungal strains was treated against various heavy metals, such as chromium, lead, mercury and cadmium (100, 200,400 & 600 ppm) (Fig 2) which pose an important threat to the soil fertility. Among the four fungal strains *Alternaria sp.* showed high heavy metal tolerance when compared with other isolates. In previous reports [23] suggest that from *Justicia adathoda,* 11 endophytic fungi were isolated and of them *Paecilomyces lilacinus* exhibit high heavy metal tolerance against lead (800 $\mu g/ml$ of metal), *Neocomospora sp.* and *Aspergillus sp*. showed very great tolerance towards copper, zinc and lead. Another report also emphasised that 158 different fungal strains were obtained from barley and soy bean and out of that 23 strains demonstrate high tolerance to cadmium [24]. Our current research focuses more on *Alternaria alternata* because, in comparison with the other two fungal isolates, it is highly tolerant of the heavy metals lead and cadmium.

3.3 Molecular Identification of *Alternaria alternata*

The fungal isolate has been identified as *Alternaria alternata* by 18S rRNA sequencing that was carried in Qbiogen Laboratory (Table 1). The heavy metal tolerant fungal isolate was subjected to 18SrRNA sequence analysis, and

the isolate was confirmed as *Alternaria alternata* (Fig 3). The isolate's raw sequence was submitted to NCBI and assigned the accession number 0Q801189. The values of the bootstrap majority consensus, obtained after 1000 replications, are represented by the figures at the branches. When two sequences are compared pairwise, the scale bar shows the genetic divergence (0.1) as the frequency of variation. (Fig 4).

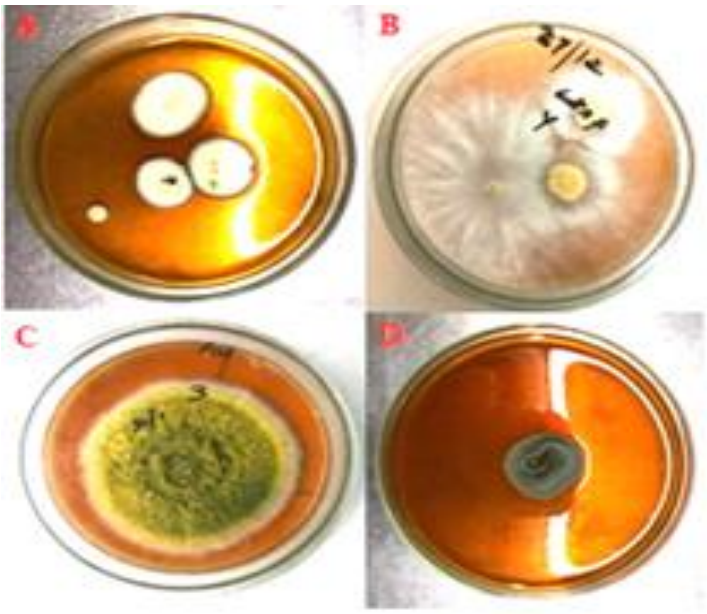
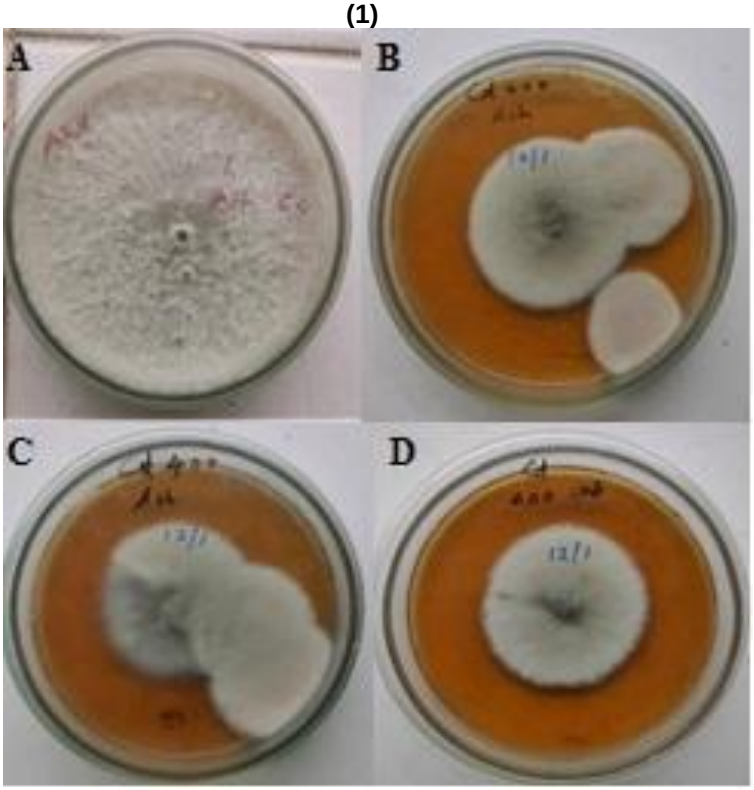


Fig. 1. Isolated fungal strains from *Solanum nigrum.* (A) *Alternaria alternata,* (B) *Aspergillus niger* (C) *Aspergillus flavus, (D) Cladosporium sp*

(1)



(2)

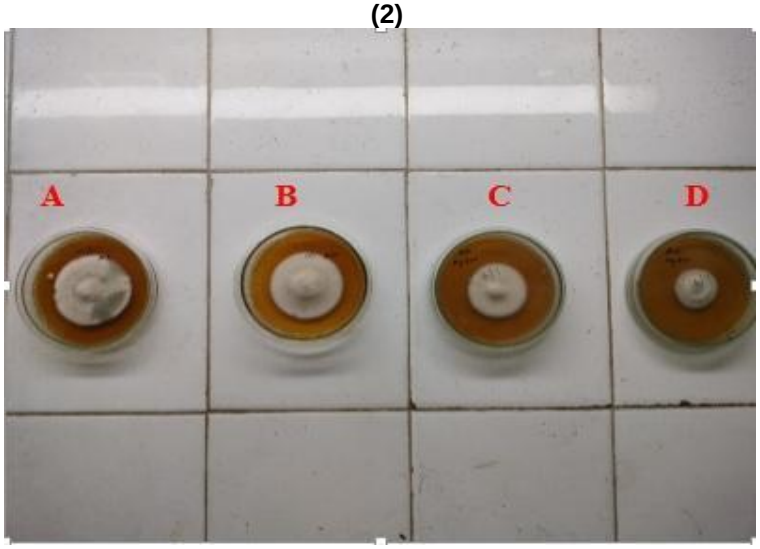


Fig. 2. The figure depicts the tolerance level of *Alternaria alternata* against various concentrations (A) 100ppm, (B) 200 ppm, (C) 400ppm, (D) 600ppm of heavy metal (1. cadmium & 2. lead)

Table 1a. The sequence of the primer used for sequencing

PRIMER NAME	PRIMER SEQUENCE
ITS1	5′- TCCGTAGGTGAACCTGCGG - 3′
ITS4	5′- TCCTCCGCTTATTGATATGC - 3′

Table 1b. PCR products of ITS region

1.	**100 bp marker**
2.	**A1**

3.4 The ITS Sequence is Represented

3.4.1 ITS sequence

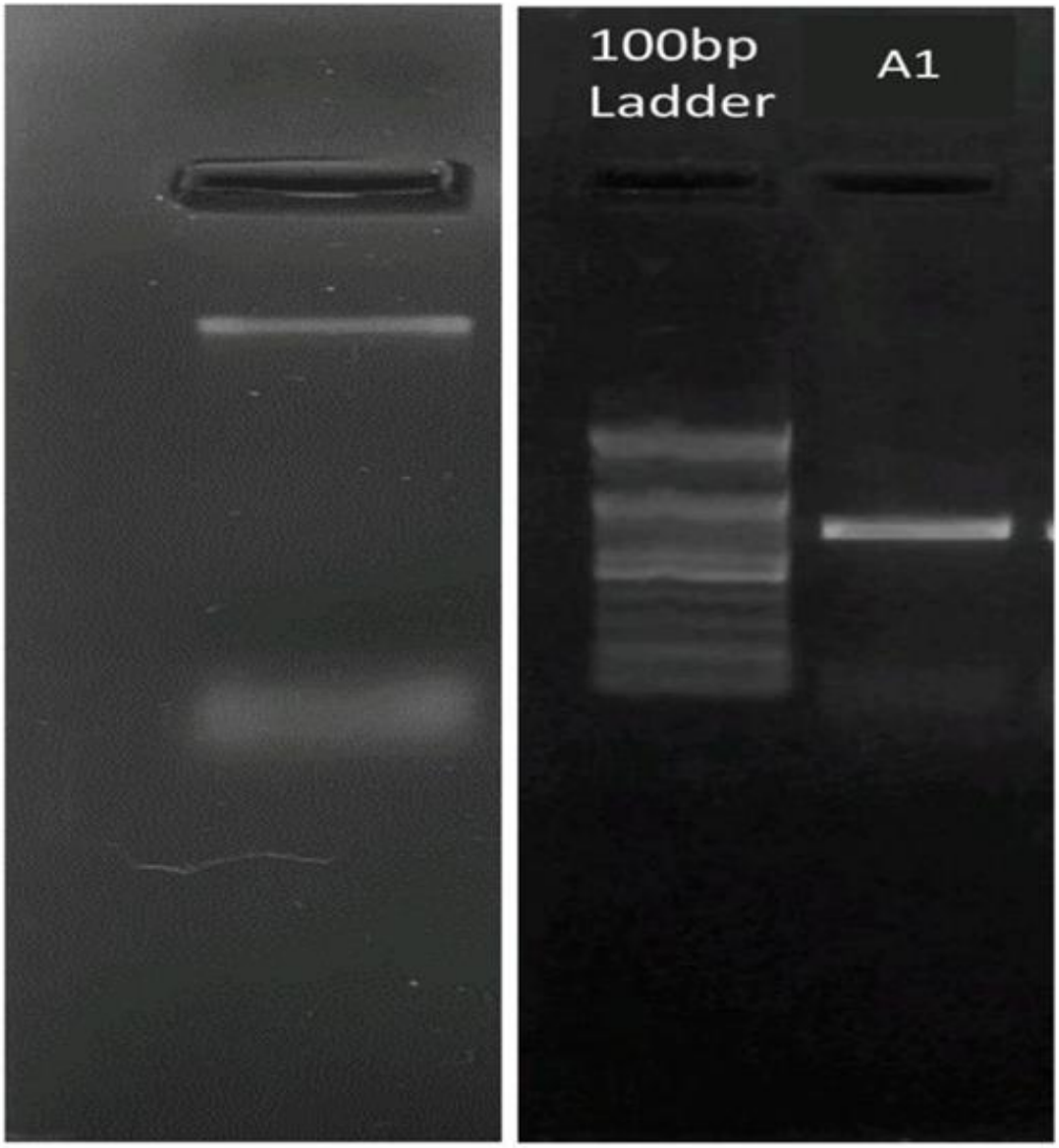
TCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGA
CAAACATAAACCTTTTGTAATTGCAATCAGCGTCA
GTAACAAATTAATAATTACAACTTTCAACAACGGAT
CTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA
AATGCGATAAGTAGTGTGAATTGCAGAATTCAGTG
AATCATCGAATCTTTGAACGCACATTGCGCCCTTT
GGTATTCCAAAGGGCATGCCTGTTCGAGCGTCAT
TTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCT
TGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTA
ATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGC
ACAAGTCGCACTCTCTATCAGCAAAGGTCTAGCAT
CCATTAAGCCTTTTTT.

3.5 Evolutionary Relationships of Taxa

The evolutionary relationship among the different species of Alternaria showed the common anestors.

3.6 Tolerance Level of *Alternaria alternata*

The tolerance level of *Alternaria alternata* was examined, the heavy metals concentration was intensified further to 1000 ppm, 3000 ppm, and 6000 ppm. The growth of *Alternaria alternata* was inhibited at this high concentration to Mercury, lead and Chromium (0.3 fungal radial growth in cm), whereas the fungi exhibit maximum resistance against Cadmium (6000 ppm with 6.1 fungal radial growth in cm).The fungal radial growth was significantly decreased with increase in the concentration of the heavy metals. Ten fungal isolates were obtained by Aishwarya et al. [7] in a prior investigation ten fungal isolates were examined for Co, Cd, Cu, and Zn. Upon initial screening, ten fungi were identified, each displaying a distinct resistance pattern against a single heavy metal.



Genomic DNA **Genomic DNA**

Fig. 3. 18SrRna Gene Sequencing of *Alternaria alternate*

3.7 MIC (Minimum Inhibitory Concentration)

The MIC Cadmium against *Alternaria alternate* showed its growth till 6000 ppm and further increasing the concentration, drastically decreased the growth of the fungi in case of Lead, the fungi exhibited its maximum growth at 2000 ppm and reduced with further increase in concentration when compared with the control.

3.8 Metal Tolerance Index (MTI)

The metal tolerance indices of the tested fungi were recorded. Growth of Alternaria alternata was recorded. The radial growth of the mycelia was examined at different concentrations of heavy metals (1000 ppm, 3000ppm and 6000 ppm). The maximum radial growth of the endophytic fungi Alternaria alternata was against the heavy metal Cadmium (0.69) followed by Lead (0.43). The fungi is too sensitive against Mercury and Chromium.

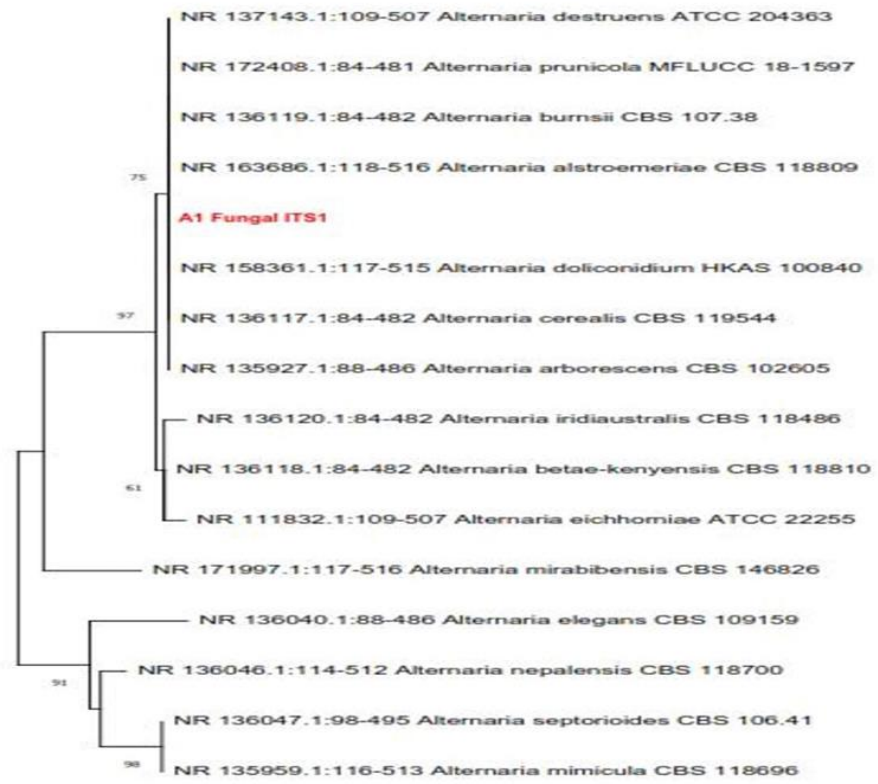
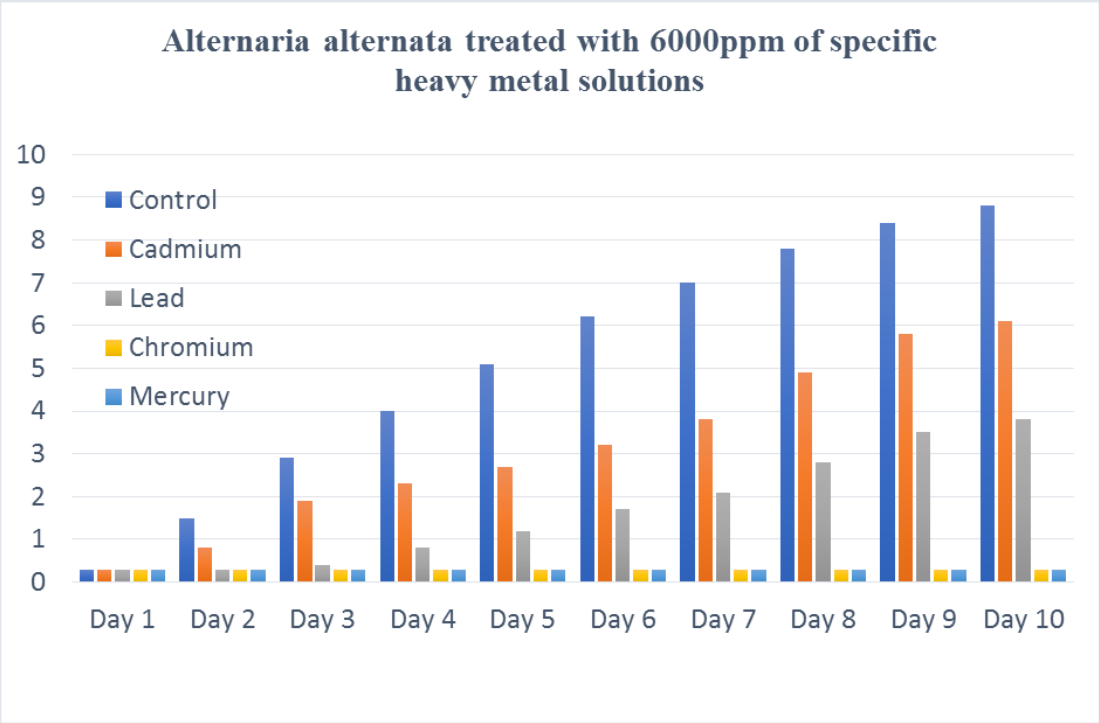


Fig. 4. A1 Fungal ITS1 identifies as *Alternaria alternate*



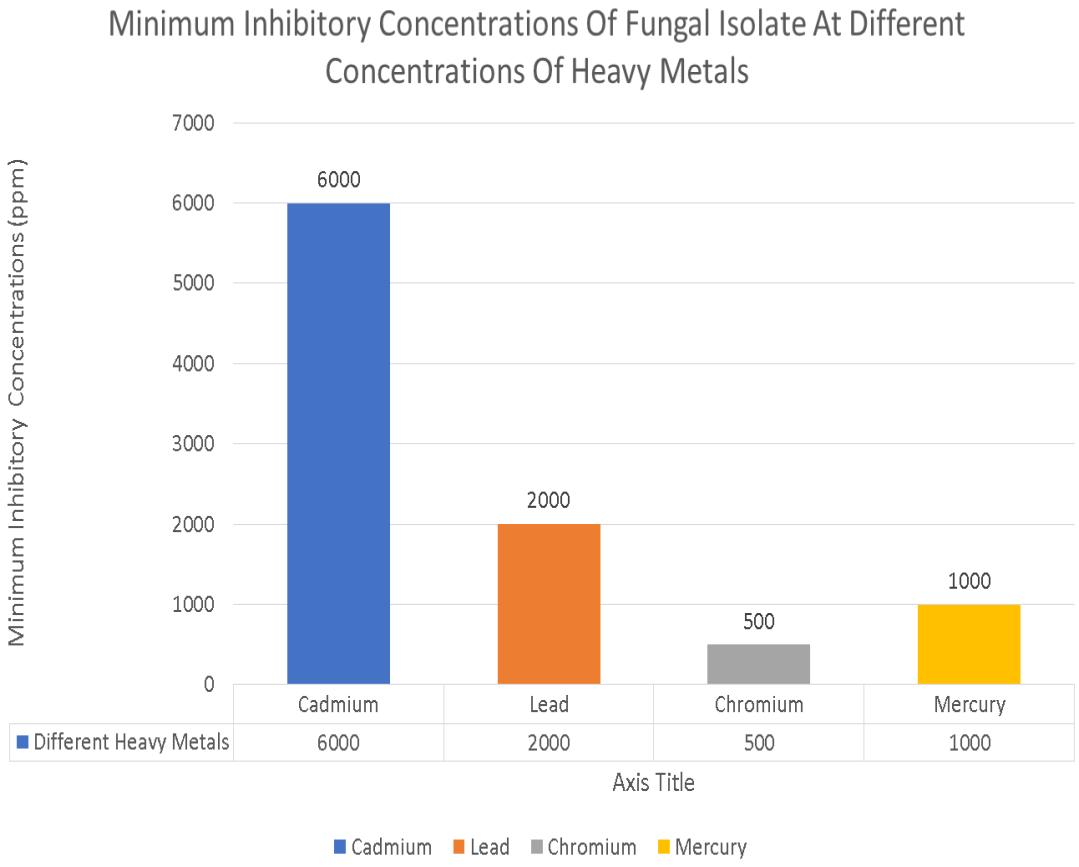
Graph 1. *Alternaria alternata* treated with 6oooppm of Cadmium and the continuous growth observed for 10 days along with PDA plate without any metal acting as control

Table 2. Radial growth of Alternaria alternata treated with Cadmium. Lead, Chromium and Mercury observed for 10 days. The radial growth in the table is expressed in centimeters (cm)

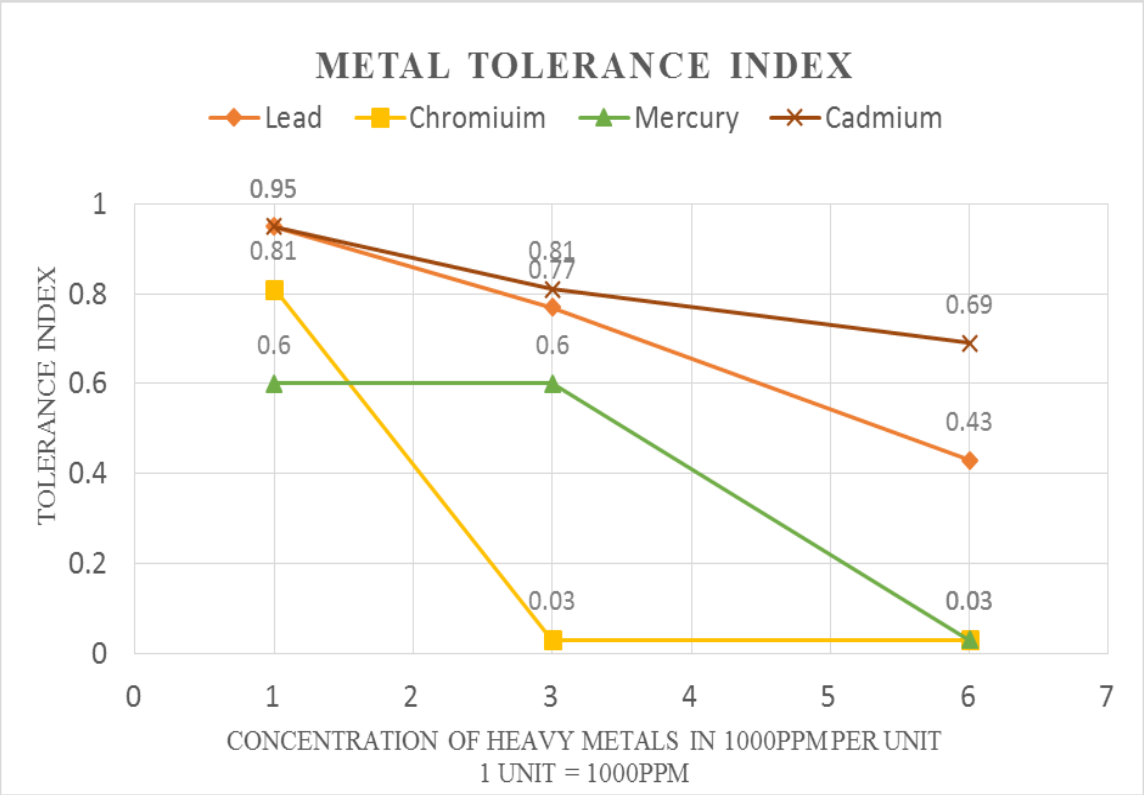
	Day									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
CONTROL	0.3	1.5	2.9	4	5.1	6.2	7	7.8	8.4	8.8
CADMIUM	0.3	0.8	1.9	2.3	2.7	3.2	3.8	4.9	5.8	6.1
LEAD	0.3	0.3	0.4	0.8	1.2	1.7	2.1	2.8	3.5	3.8
CHROMIUIM	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
MERCURY	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3

3.9 Bioaccumulation of the Heavy Metals in the Liquid Media

Alternaria alternata in aqueous growth media (i.e.) potato dextrose broth containing metal solutions was used to carry out the metal accumulation process. In an aqueous growth media, the tolerance and accumulation of the heavy metals (cadmium, lead, mercury, and chromium) were assessed. The heavy metal tolerance of the fungus, measured in parts per million, was in the following order: cadmium, lead, mercury, chromium, in the concentration of 600 ppm. The greater concentration of heavy metal reduced the growth of the isolate. In heavy metals bioaccumulation study, *Alternaria alternata* showed high tolerance at Cadmium (89%) followed by Lead (50%), Mercury (54%) and Chromium (12%).



Graph 2. Minimum inhibitory concentrations of heavy metals at different concentrations in ppm



Graph 3. Metal Tolerance Index of *Alternaria alternata* to different concentrations of Heavy Metals

Table 3. Metal Tolerance Index with respect to different concentrations of heavy metals (ppm)

Heavy Metals	TI at 1000ppm	TI at 3000ppm	TI at 6000ppm
Cadmium	0.95	0.81	0.69
Lead	0.95	0.77	0.43
Chromium	0.81	0.03	0.03
Mercury	0.6	0.6	0.03

3.10 Toxigenicity Testing

Earthworms moved swiftly and ferociously at first, perhaps as a result of switching from a terrestrial to an aquatic home, but they appeared to be acting normally and moving normally during the control trials. After being observed for 24 hrs, earthworms from control trials showed no morphological alterations or injury. The control experiment and the individual metal solutions were carried out simultaneously. Earthworms were introduced to the known metal solution concentration. The earthworms reacted with extremely erratic restless movements that gradually subsided when they were initially placed in the aqueous cadmium solution. The earthworms eventually showed signs of clitellum enlargement and slow movements. Upon immersion in a solution containing 600 parts per million of cadmium, the worm immediately exhibited signs of restlessness. The body expanded with a swollen clitellum and turned pale and rigid after an initial effort. The worm's body became long and curled after an hour of exposure, until it finally perished. Additionally, segment fragmentation was seen in the posterior part of the body. Because heavy metals are found in their free form in environments with moisture, earthworms are often susceptible to them. Based on the worms' quick morphological changes and the metal build up in their various tissues, cadmium metal was shown to be toxic to them since they perished from it in a matter of minutes. The earthworm began to move slowly and behave weirdly.

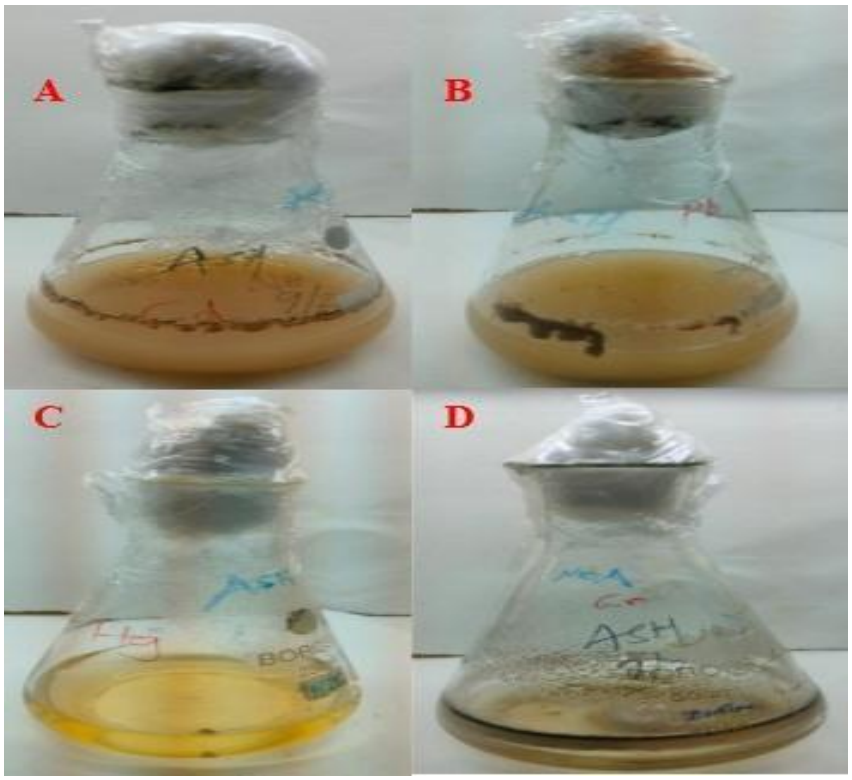


Fig. 5. Bioaccumulation of *Alternaria alternata* in Potato Dextrose Broth treated with heavy metals

Fig. 6. The toxigenicity test of Earthworm (A) Earthworm Control, (B) Earthworm treated with water, (C) Earthworm treated with Cadmium (600ppm)

The earthworm that was exposed to distilled water stayed the same. The earthworms turned dull and died within an hour after being exposed to cadmium. Prior research clearly shows that both ingestion and integumentary absorption may contribute to earthworm mortality in aqueous metal solutions [25]. Earthworms were identified as biomarkers in earlier research, and these markers can be applied to treatment methods including sludge and wastewater treatment [26].

3.11 Statistical Analysis

The information was shown as the average across three duplicates, along with a SD (standard deviation). Graph Pad Prism (Version 5.03, CA, USA) was utilized for statistical analysis.

4. CONCLUSION

Heavy metals cannot be broken down into innocuous compounds and will always remain in the environment; their release into the environment has been steadily rising due to industrial activity and technological advancement. This poses a serious risk to the soil health and environment. The use of biological methods holds significant promise in aiding phytoremediation by facilitating the bioaccumulation of heavy metals through the use of endophytic fungus. High capacity for the biosorption and accumulation of heavy metals from metal-contaminated soils were demonstrated by the endophytic fungus. The heavy metal tolerance of *A. alternata* biomass was tested for eliminating cadmium, lead, chromium, and mercury, compared to *Cladosporium sp., Aspergillus niger*, and *Aspergillus flavus*. *Alternaria alternata*, a high-heavy metal-resistant fungal isolate, had a higher tolerance level. According to the data, a significant amount of heavy metals were eliminated by the fungus, which will considerably contribute to the environment being free of harmful heavy metals. This study indicated that the ability of the isolated fungi to bio accumulate and remove heavy metals from contaminated sites. This study proposes the possibility of the use of this fungi for heavy metal bioremediation possible. As a result, when crops are susceptible to disease, it will be important to look at the spectrum of metal tolerance that fungi exhibit when developing fungicide formulations and to analyse studies that centre on the interaction between metal and pathogen. This investigation clearly indicates the potential involvement of *Alternaria alternata* in heavy metal bioremediation.

5. SUMMARY

The main objectives of the study are to isolate an effective endophytic fungi from the plants, and to investigate the resistance of the isolated fungi to various heavy metals, and ascertain whether the heavy metal is toxic to earthworms. The fungal isolate *Alternaria alternata*, which was resistant to heavy metals and exhibited a greater degree of tolerance. Comparing the cadmium-treated Earthworm to the control group, the toxigenicity test showed that the treated Earthworm became immobile a few minutes after being exposed to the metal. Thus, the study unequivocally shows that endophytic fungi are beneficial for maintaining soil fertility and reducing environmental pollution.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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